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Up-regulation of a photosystem II core protein phosphatase inhibitor and sustained D1 phosphorylation in zeaxanthin-retaining, photoinhibited needles of overwintering Douglas fir

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ABSTRACT

Overwintering needles of the evergreen conifer Douglas fir exhibited an association between arrest of the xanthophyll cycle in the dissipating state (as zeaxanthin + antheraxanthin; Z + A) with a strongly elevated predawn phosphorylation state of the D1 protein of the photosystem II (PSII) core. Furthermore, the high predawn phosphorylation state of PSII core proteins was associated with strongly increased levels of TLP40, the cyclophilin-like inhibitor of PSII core protein phosphatase, in winter versus summer. In turn, decreases in predawn PSII efficiency, F_v/F_m, in winter were positively correlated with pronounced decreases in the non-phosphorylated form of D1. In contrast to PSII core proteins, the light-harvesting complex of photosystem II (LHCII) did not exhibit any nocturnally sustained phosphorylation. The total level of the D1 protein was found to be the same in summer and winter in Douglas fir when proteins were extracted in a single step from whole needles. In contrast, total D1 protein levels were lower in thylakoid preparations of overwintering needles versus needles collected in summer, indicating that D1 was lost during thylakoid preparation from overwintering Douglas fir needles. In contrast to total D1, the ratio of phosphorylated to non-phosphorylated D1 as well as the levels of the PsbS protein were similar in thylakoid versus whole needle preparations. The level of the PsbS protein, that is required for pH-dependent thermal dissipation, exhibited an increase in winter, whereas LHCII levels remained unchanged.

Key-words: D1; energy dissipation; phosphorylation; photosynthesis; photoprotection; PsbS; TLP40; winter stress; zeaxanthin.

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INTRODUCTION

Many sun-exposed evergreen species that cease growth under winter stress down-regulate the capacity for photosynthesis and up-regulate the capacity of photoprotection (Oberhuber & Bauer 1991; Ottander & Öquist 1991; Ottander, Campbell & Öquist 1995; Verhoeven, Adams & Demmig-Adams 1999a; Adams *et al*. 2001a, b, 2002, 2004; Matsubara *et al*. 2002; Öquist & Huner 2003; Ensminger *et al*. 2004). Up-regulation of photoprotection occurs in the form of increased levels of pigments involved in photoprotective dissipation of excess excitation energy as heat, namely the xanthophyll cycle carotenoids and lutein (Demmig-Adams & Adams 1996; Niyogi 2000). Even on milder winter days, these evergreen species can maintain the xanthophyll cycle in the state that is highly converted to the photoprotective pigments zeaxanthin and antheraxanthin (Z + A) and exhibit sustained decreases in the efficiency of the conversion of solar energy into photosystem II (PSII) photochemistry (as PSII efficiency F_v/F_m from chlorophyll fluorescence; Adams & Demmig-Adams 1994, 1995; Adams *et al*. 1995, 2001a, b, 2002; Ottander *et al*. 1995; Verhoeven, Adams & Demmig-Adams 1996, 1998; Adams & Barker 1998; Logan *et al*. 1998; Verhoeven *et al*. 1999a; Gilmore & Ball 2000; Matsubara *et al*. 2002). Down-regulation of photosynthetic electron transport capacity in overwintering evergreen species has, furthermore, been reported to be associated with decreased levels of D1 protein and total PSII cores whereas the light-harvesting chlorophyll complexes are largely preserved (Ottander *et al*. 1995; Adams *et al*. 2001b; Ensminger *et al*. 2004). Inactivation of PSII photochemistry and sustained high levels of photoprotective thermal energy dissipation are both likely to contribute to the observed decreases in the efficiency of solar energy conversion in PSII.

In the present study, D1 phosphorylation state, Z + A retention, and sustained down-regulation of PSII efficiency were assessed in an evergreen species, the conifer Douglas fir, that down-regulates photosynthesis in the winter. Major thylakoid proteins subject to phosphorylation include the

PSII reaction centre proteins D1, D2, CP43, and a 9-kDa subunit, as well as LHCII and CP29 (Vener, Ohad & Andersson 1998). D1/D2 become phosphorylated in high light (Rintamäki *et al*. 1997; Ebbert *et al*. 2001) and dephosphorylation is accelerated at elevated temperature (Rokka *et al*. 2000). A sustained high phosphorylation state of the D1 protein is maintained overnight in photoinhibited leaves of a rainforest evergreen subjected to a sudden transfer to high light levels (Ebbert *et al*. 2001). Dephosphorylation of PSII core proteins is inhibited by a recently characterized regulatory protein (TLP40 for thylakoid lumen protein of 40 kDa). TLP40 exhibits reversible binding to the thylakoid membrane (Vener *et al*. 1999), inhibits PSII core protein dephosphorylation in the thylakoid-bound state, and becomes released from the thylakoid membrane at high temperature (Rokka *et al*. 2000). The role of D1 phosphorylation is still largely unknown (Booij-James *et al*. 2002). Thylakoid protein phosphorylation is thought to play a role in regulating protein turnover (Ebbert & Godde 1996; Salonen, Aro & Rintamäki 1998; Yang *et al*. 1998; Baena-Gonzalez, Barbato & Aro 1999), protein function (see, e.g. Allen 1992), and possibly signal transduction (Vener *et al*. 1998; Misra & Biswal 2000).

MATERIALS AND METHODS

Field collections

Pseudotsuga menziesii (Mirbel) Franco (Douglas fir) growing in Boulder, CO (40°0.8′N, 105°16.8′W, elevation of 1730 m), was characterized in summer 2004, winter of 2001, and on four dates during 1999–2000. The efficiency of solar energy conversion was determined as F_v/F_m or F_v'/F_m' (using a PAM-2000 chlorophyll fluorometer; Walz, Effeltrich, Germany; see Demmig-Adams *et al*. 1996) from needles under the ambient conditions prior to sunrise (predawn) and/or at midday during exposure to full sunlight [incident photon flux density (PFD) ranged from 1800 to 2050 μmol photons m^{-2} s^{-1}; measured with an LI-185B meter and LI-190SB sensor; Li-Cor, Lincoln, NE, USA]. In addition, self-shaded needles within the north side of the Douglas fir canopy (incident PFD ranging from 50 to 60 μmol photons m^{-2} s^{-1} at midday) were characterized. Additional, comparable needles were simultaneously collected and immediately frozen in liquid nitrogen for subsequent extraction and characterization of thylakoid pigments (Adams & Demmig-Adams 1992) and proteins.

Predawn and midday needle temperatures were characterized with a thermocouple thermometer (TH-65 meter; Wescor, Logan, UT, USA). During 1999–2000, Douglas fir needle temperatures for south-facing needles, predawn/midday, were 21.9 °C/36.1 °C (27 July), –3.5 °C/17.3 °C (11 December), 0.5 °C (16 January), and –7.3 °C/4–5.5 °C (28 and 29 January) and for north-facing needles were 21.9 °C/28.9 °C (27 July), –3.5 °C/1.8 °C (11 December), 0.5 °C (16 January), and –7.3 °C/–0.6 °C (28 and 29 January). Characterization in 2001 occurred on 20 January, with a predawn needle temperature of –3.2 °C. For comparison, the 10 d prior to collection on 29 January 2000 experienced a mean minimum air temperature of –3.5 ± 2.3 °C and a mean maximum air temperature of 7.0 ± 4.4 °C, whereas for 20 January 2001 the mean minimum air temperature was –8.0 ± 4.5 °C and the mean maximum air temperature was 5.5 ± 5.2 °C. Needles were also characterized on 6 July 2004, with a predawn needle temperature of 11.2 °C.

Protein extraction and characterization

Proteins were extracted from needles, either in a single step or subsequent to thylakoid isolation (after Ottander *et al*. 1995), with the following modifications.

To extract proteins in a single step, needles were ground in liquid nitrogen with a Kontes Duall Comp SZ 22 tissue grinder (Kontes Glass Company, Vineland, NJ, USA), followed by the addition of 1 mL of ice-cold isolation buffer [50 mM Tricine NaOH (pH 7.8), 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, and 20% (w/v) polyethyleneglycol PEG 3350], also including 50 mM of the protein phosphatase inhibitor sodium fluoride as well as 12 and 2.3 mM of the protease inhibitors 6-aminocaproic acid and benzamide, respectively. The sample was then kept on ice for 15 min, after which an equal volume of sample buffer, as used in Elich, Edelman & Mattoo (1992), was added. The sample was then mixed using a vortex mixer (Fisher Vortex Genie 2; Scientific Industries, Inc., Bohemia, NY, USA), kept for 15 min at room temperature, and finally heated at 95 °C for 7 min.

During thylakoid isolation, the same initial step of needle grinding and addition of isolation buffer (4 instead of 1 mL) was used as described above for the one-step procedure. However, instead of keeping the resulting sample in ice for 15 min, the homogenate was filtered through one layer of nylon mesh (Spectra/Mesh Nylon filter; Spectrum, Laguna Hills, CA, USA) and centrifuged for 3 min at 6000 *g*. The resulting pellet was washed with 50 mM Tricine NaOH (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$, and re-suspended in 2 mL modified ice-cold isolation buffer. The sample was then centrifuged for 3 min at 6000 *g*, the pellet was washed again, and re-suspended again in ice-cold isolation buffer. To remove larger particles, the sample was centrifuged for 30 s at 300 *g* and the supernatant transferred to a new vial and centrifuged for 3 min at 6000 *g*. The pellet was re-suspended in re-suspension buffer containing 0.1 M sorbitol, 50 mM Tricine NaOH (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$, centrifuged for 3 min at 4000 *g*, and re-suspended in re-suspension buffer. After a final centrifugation step for 3 min at 4000 *g*, the pellet was kept on ice, followed by solubilization of the thylakoid membranes.

For the characterization of proteins extracted from whole needles or isolated thylakoids, a protocol described by Elich *et al*. (1992) was used. Proteins were separated on a 10 to 20% (w/v) acrylamide gradient gel using Bio-Rad Protean II xi equipment (Bio-Rad, Hercules, CA, USA), and samples from a given experiment were loaded on the gel on an equal chlorophyll basis of 1.4 μg chlorophyll.

Immunoblotting was carried out for at least 6 h at 0.2 mA using Protran BA79 nitrocellulose membranes (Schleicher

& Schuell BioScience, Dassel, Germany). For immunodetection, alkaline phosphatase-conjugate was used (170–6518; Bio-Rad or 61–3122; Zymed Laboratories, South San Francisco, CA, USA). Optical densities of immunodetection signals were determined using Powerlook II equipment (Umax Technologies, Inc., Fremont, CA, USA) and software 'Intelligent Quantifier' (Bio Image, Ann Arbor, MI, USA).

The following primary antibodies were used: antiphosphothreonine (Rabbit antiphosphothreonine 71–8200; Zymed Laboratories, Inc.); anti-Lhcb2 (Fig. 3F) and anti-Lhcb (4778) (Fig. 3E) and anti-OEC (33 kDa) (Sigrist & Staehelin 1994); anti-TLP40 (Fulgosi *et al*. 1998); anti-PsbS, anti-D1 (chicken anti-PsbS and chicken anti-PsbA; Agrisera AB, Vännäs, Sweden); and anti-D1 (SP1 and SP2) (Booij-James *et al*. 2002). Anti-D1 (SP1) recognizes only the non-phosphorylated form of D1 and not the phosphorylated region, whereas anti-D1 (SP2) recognizes both forms (phosphorylated and non-phosphorylated) of D1.

Statistical analyses

To test for significant differences among several means, analysis of variance was performed followed by a Tukey–Kramer comparison for honestly significant differences. For linear relationships, correlation coefficients (r^2) of linear fit were determined. For comparisons between two means, a Student's *t*-test was applied. All statistical analyses were done using JMP Statistical Software (SAS Institute Inc., Cary, NC, USA). All errors are provided as standard deviations.

RESULTS

Douglas fir needles were collected on a warm summer day and a cold winter day. Overwintering needles exhibited sustained depressions of predawn PSII efficiency (F_v/F_m of 0.842 ± 0.003 in summer and 0.132 ± 0.022 in winter) and overnight retention of the photoprotective xanthophylls zeaxanthin and antheraxanthin ($Z + A$ of 8.3 ± 0.4 in summer and 144.2 ± 14.3 mmol mol^{-1} chl in winter). Proteins were extracted from these needles via parallel single-step extractions of whole needles versus standard protein extraction from thylakoid preparations (Figs 1 & 2). Chloroplast proteins quantified included the PsbS protein (Fig. 1a & b), the D1 protein of PSII cores (Fig. 1c–f) and its phosphorylation state (Fig. 2a–d), the oxygen-evolving complex (OEC; Fig. 1g & h), and the TLP40 protein (Fig. 2e & f). Summer to winter ratios of PsbS and of phosphorylated and non-phosphorylated D1 were similar irrespective of whether whole needle or thylakoid preparations were used (Figs 1a, b & 2a–d). D1 exhibited an elevated phosphorylation state at predawn in winter (evident from an increase in antiphosphothreonine immunoreactivity; Fig. 2c & d) and a corresponding decrease in the level of non-phosphorylated D1 (evident from a decrease in SP1 antibody labelling that only recognizes the unphosphorylated form of D1; Fig. 2a & b). The fact that the changes in these two independent parameters for phosphorylated and non-phosphorylated D1 were mirror images of each other is solid support for these changes in D1 phosphorylation state at predawn.

The total level of the D1 protein (assessed via two different antibodies; Fig. 2a & b) was the same in whole leaf preparations but not in thylakoid preparations where less D1 was recovered in winter (Fig. 1c–f). A similar, albeit smaller, difference between preparations was evident for OEC (Fig. 1g & h). This indicates that D1 and, to a lesser extent, OEC, is lost during thylakoid preparation from overwintering Douglas fir needles. Both phosphorylated and non-phosphorylated forms of D1 are apparently lost in equal proportions during thylakoid preparation since the summer to winter ratio of phosphorylated to non-phosphorylated D1 was similar in whole needle versus thylakoid preparations (Fig. 2a–d).

The protein kinase responsible for phosphorylation of PSII core proteins could not be addressed directly via an immunological approach. However, an antibody against the TLP40 protein, that acts as an inhibitor of PSII core protein phosphatase, exhibited a good reaction in Douglas fir (Fig. 2e & f). The level of TLP40 increased dramatically in overwintering needles. This increase was less pronounced in thylakoid preparations versus whole needle preparations, which indicates that relatively more TLP40 is lost during thylakoid preparation in winter versus summer.

The level of PsbS, the protein required for the rapidly reversible, pH-dependent form of xanthophyll cycle-dependent thermal dissipation (Li *et al*. 2000, 2002; Külheim, Agren & Jansson 2002), was also greater in the winter relative to the summer (Fig. 1a & b) using either extraction method.

Additional collections were made in other years and included both subfreezing and above-freezing days in winter. Furthermore, since sun-exposed needles of Douglas fir experience a considerable level of excess light even during the summer, a characterization of needles from the north side of the Douglas fir canopy was added to more fully examine the impact of winter conditions (Fig. 3). Both sun-exposed (south-facing) and self-shaded (north-facing) needles of Douglas fir exhibited significant winter decreases in both the midday and predawn levels of maximal PSII efficiency (Fig. 3A & B). Incident PFDs on the north-facing needles at midday were low and typically ranged between 50 and 60 μmol photons m^{-2} s^{-1} throughout the year. Nonetheless, midday PSII efficiency (F_v'/F_m') was reduced by approximately 50% on the cold day in December (compared to summer) and to less than 0.2 on the cold day in January (Fig. 3B), and the predawn efficiency of solar energy conversion into PSII photochemistry (F_v/F_m) exhibited depressions that reached low levels of 0.4 by the end of January (Fig. 3B). Predawn and midday levels of $Z + A$ also increased in winter compared to summer (Fig. 3C & D).

D1 phosphorylation was enhanced under winter conditions, both during the day and sustained throughout the night (Fig. 3E & F). The level of predawn PSII efficiency (F_v/F_m) was thus positively correlated with the predawn

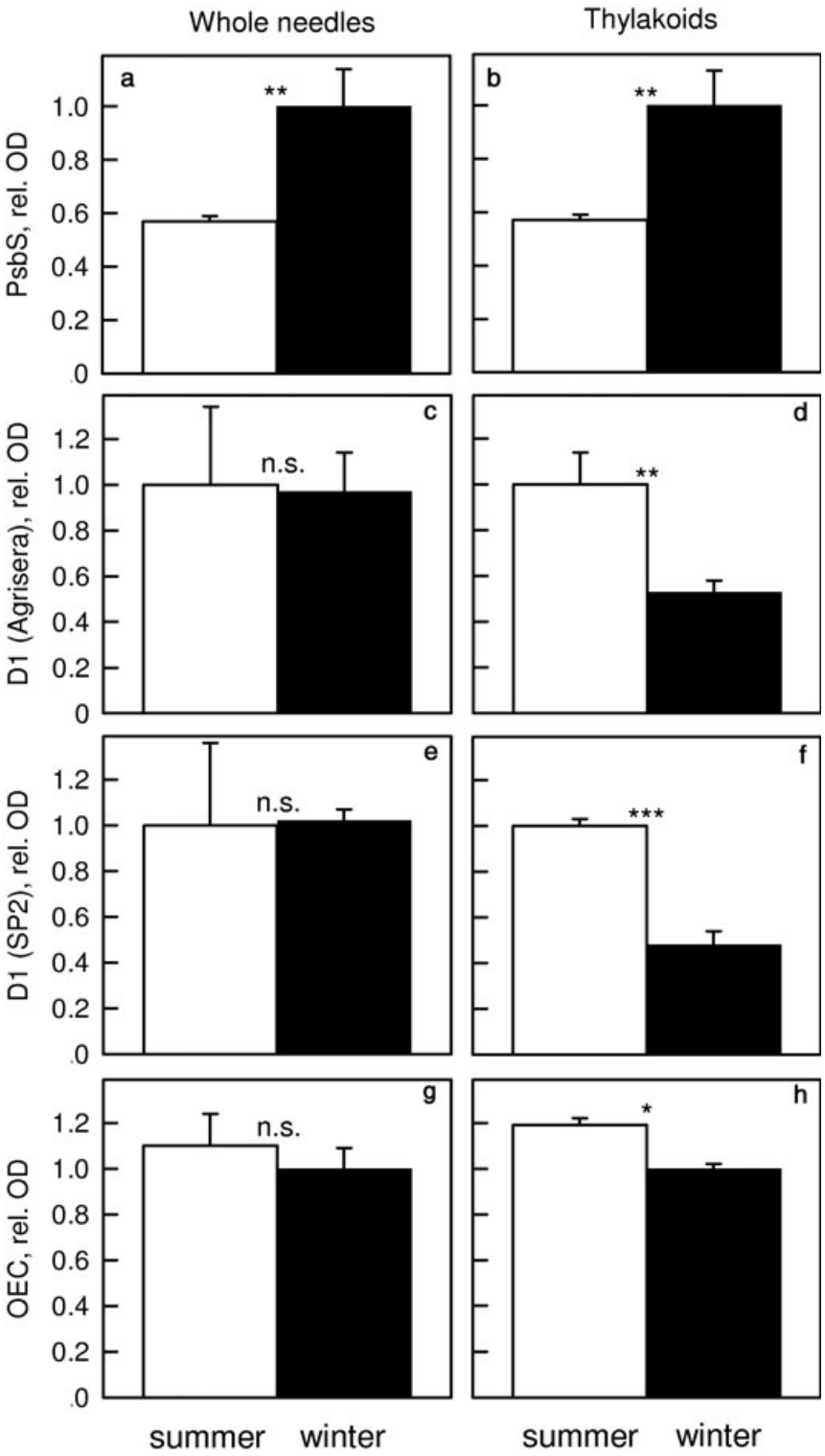

Figure 1. Relative levels of (a, b) the PsbS protein, (c–f) the D1 protein, and (g, h) the oxygen-evolving complex (OEC) in needles of Douglas fir collected predawn on a warm summer day (6 July 2004; open bars) and a subfreezing winter day (20 January 2001; filled bars). Proteins were extracted in either a single step from whole needles (left panels) or from thylakoids prepared from these needles (right panels). Immunodetection was with appropriate antibodies, including two different ones (Agrisera and SP2) for the D1 protein. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n.s. = not significantly different; $n = 3$. For further details, see Material and Methods.

level of non-phosphorylated D1 (Fig. 4a) and negatively correlated with the predawn level of phosphorylated D1 (Fig. 4c). Thus, photoinhibition, in the form of sustained decreases in predawn PSII efficiency, was closely and positively correlated with an increasing level of sustained D1 phosphorylation. Conversely, the level of predawn Z + A retention was negatively correlated with the predawn level of non-phosphorylated D1 (Fig. 4b) and positively correlated with the predawn level of phosphorylated D1 (Fig. 4d). In addition to nocturnal retention of a high phosphorylation state of D1 under winter conditions (Fig. 3E & F), a protein likely to be D2 (based upon its position) as well as a protein in the 43 kDa range exhibited strong nocturnally sustained phosphorylation on the cold nights (not

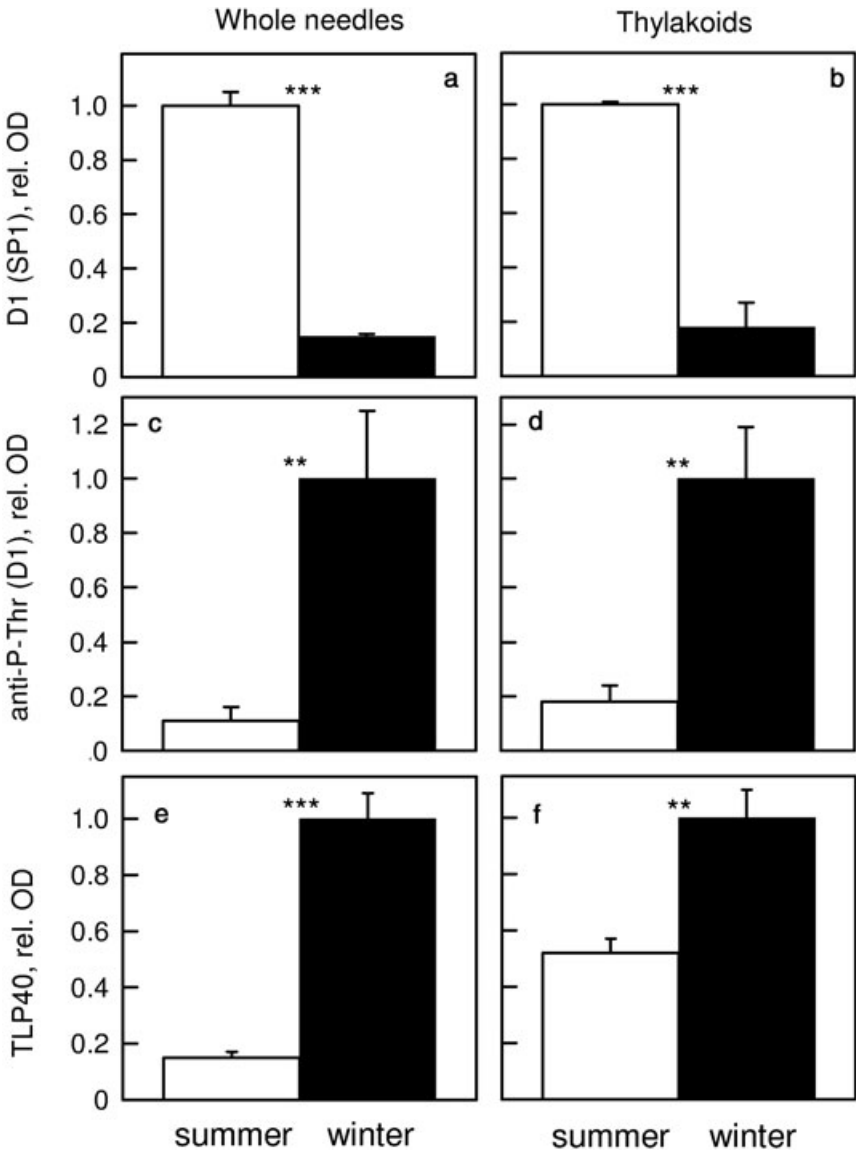

Figure 2. Relative levels of (a, b) the non-phosphorylated form of the D1 protein, (c, d) D1 phosphorylation, and (e, f) the protein TLP40, an inhibitor of PSII core protein phosphatase, in needles of Douglas fir collected predawn on a warm summer day (6 July 2004; open bars) and a subfreezing winter day (20 January 2001; filled bars). Proteins were extracted either in a single step from whole needles (left panels) or from thylakoids prepared from these needles (right panels). Immunodetection was with SP1 for non-phosphorylated D1, anti-TLP40 for TLP40, and antiphosphothreonine for phosphorylated D1. $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 3$. For further details, see Material and Methods.

shown). Although antibodies to confirm the identity of these latter two proteins in Douglas fir were not available, the latter protein is most likely the PSII core antenna protein CP43. Both D2 and CP43 are known to be subject to regulation by phosphorylation (Bennett 1991), presumably by the same kinase/phosphatase system that modulates D1 (Rokka *et al*. 2000). No phosphorylation of LHCII, CP29, or the lower molecular weight proteins at 9 or 12 kDa was detectable at either predawn or noon in any season (not shown). LHCII phosphorylation is normally strongest at very low (limiting and non-excessive) PFDs, and absent or greatly diminished in darkness and at higher PFDs (Cleland *et al*. 1990; Haldrup *et al*. 2001; Martinsuo *et al*. 2003), although persistent LHCII phosphorylation in darkness can occur in some species grown under very low PFD (Ebbert *et al*. 2001). The antiphosphothreonine antibody has been shown to be an adequate means of assessing thylakoid phosphorylation, including LHCII, in a number of species (Rintamäki *et al*. 1997; Pursiheimo *et al*. 1998; Ebbert *et al*. 2001), and readily detected LHCII phosphorylation under warm conditions in our hands (not shown). The sustained high nocturnal D1 (PSII core protein) phosphorylation state (Figs 1c, d & 3E, F) in the absence of LHCII phosphorylation at predawn in overwintering Douglas fir was thus clearly a differential physiological response to winter conditions.

Both sun-exposed and shaded Douglas fir needles exhibited trends for higher winter levels of the PsbS proteins, although the levels of LHCII did not change with season when expressed on a chlorophyll basis (Fig. 3E & F).

DISCUSSION

A key finding of this study is the correlation among decreases in the efficiency of solar energy conversion in PSII, de-epoxidation of the xanthophyll cycle, and a sus-

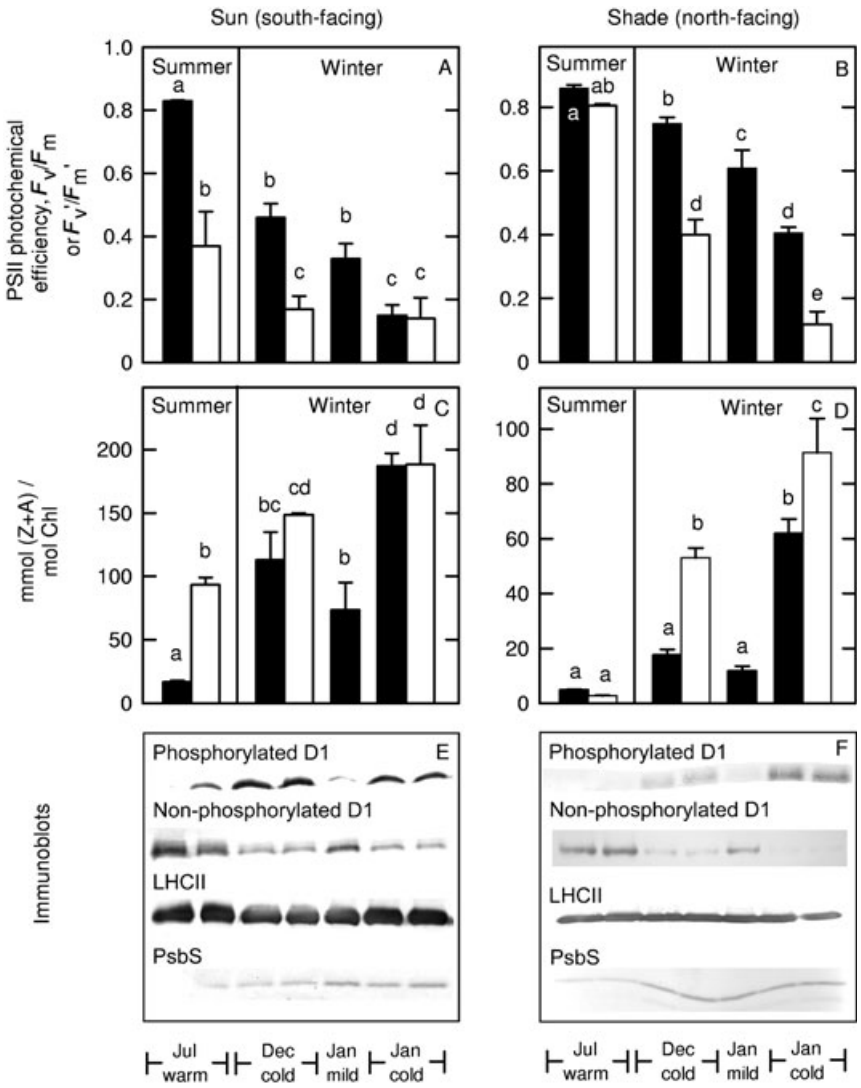

Figure 3. Pre-dawn (filled bars) and midday (open bars) (A, B) efficiency of solar energy conversion in PSII (F_v/F_m predawn or F_v'/F_m' at midday), (C, D) levels of zeaxanthin + antheraxanthin (Z + A) relative to total chlorophyll, and (E, F) corresponding immunoblots with antiphosphothreonine antibody (anti-P-Thr; detecting phosphorylated D1), non-phosphorylated D1 (SP1), LHCII or the LHCII protein Lhcb2, and PsbS in sun-exposed, south-facing (left panels) and shaded, north-facing (right panels) needles of Douglas fir during 1999–2000. Means ±SD are depicted for PSII efficiency ($n = 3$–5) and Z + A levels ($n = 3$), and significant differences are indicated by lower case letters at the $P < 0.05$ level. Predawn and midday levels of proteins are shown for July, December, and a cold day in January, and predawn levels only on a milder day in January. All proteins were extracted from isolated thylakoids. Protein extraction and immunoblotting for north-facing needles and for PsbS from south-facing needles was as described in Material and Methods, whereas for phosphorylated D1, non-phosphorylated D1, and LHCII from south-facing needles a procedure by Ebbert *et al.* (2001) was followed, and samples were loaded on gels on an equal chlorophyll basis of 6 μg chlorophyll. For immunodetection of the latter three proteins, horseradish peroxidase-conjugate was used (170–6515, Bio-Rad).

tained high phosphorylation level of the D1 protein. Specifically, decreases in predawn PSII efficiency (photoinhibition) were positively correlated with decreases in non-phosphorylated D1 and negatively correlated with increased levels of phosphorylated D1. In turn, predawn PSII efficiency was inversely correlated with Z + A retention, and Z + A retention was thus greater when D1 phosphorylation was greater and the level of non-phosphorylated D1 was decreased. In overwintering needles of Douglas fir, both the xanthophyll cycle and the D1 protein are apparently arrested in their 'high-light states', with high Z + A and D1 phosphorylation, throughout the day–night cycle.

What could be the significance of the correlation between Z + A retention and modification of PSII cores by sustained phosphorylation? As was previously shown by Verhoeven *et al.* (1999b), some of the retained Z + A may be present in PSII core complexes of leaves exposed to low temperatures. It has been suggested that PSII core/inner antenna proteins are rearranged into dissipating complexes in overwintering evergreens with sustained low PSII efficiencies (Gilmore *et al.* 2003). One may speculate that such a restructuring of PSII cores into thermally dissipating centres that incorporate Z + A is facilitated by a sustained high phosphorylation state of D1.

One may also speculate that sustained D1 phosphorylation may prevent turnover of PSII cores and replacement of protected, highly dissipating centres with unprotected photochemically competent centres (Salonen *et al.* 1998). D1 dephosphorylation is thought to be a prerequisite for degradation and replacement of D1 (Baena-Gonzalez *et al.* 1999), or phosphorylation may be a means of maintaining the status quo regardless of whether the D1 molecule has been tagged for turnover or not. In addition, phosphorylation of PSII core proteins may play a role in the inactivation of photochemistry and/or up-regulation of thermal dissipation itself. It has been suggested that over the course of evolution, reversible phosphorylation of D1 may have replaced multiple D1 genes in cyanobacteria (Booij-James *et al.* 2002). In the latter group, transition to a different D1

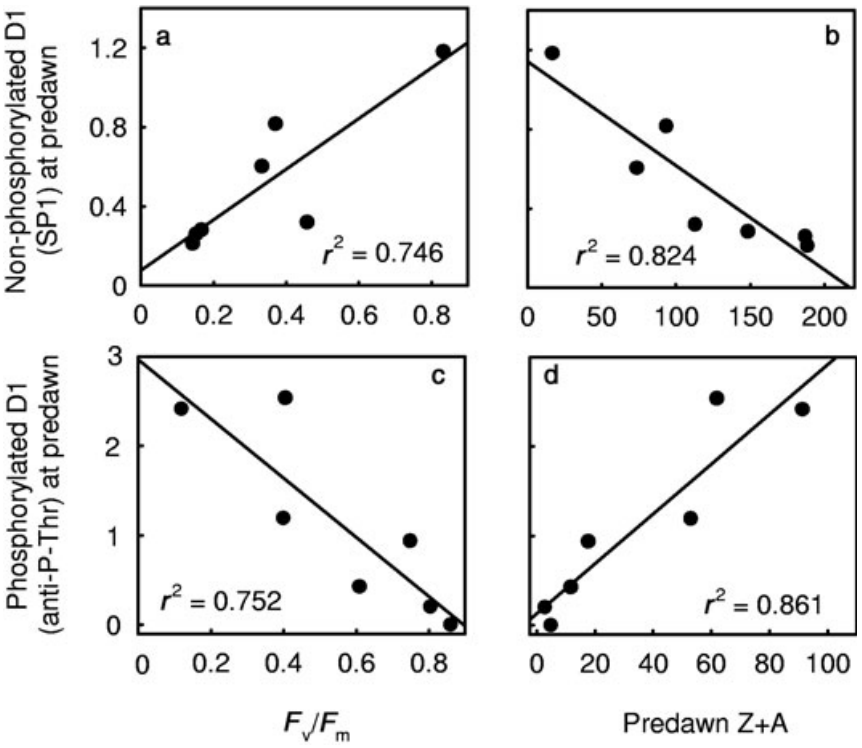

Figure 4. Correlations between either (a, c) predawn PSII efficiency F_v/F_m and aspects of D1 phosphorylation state or (b, d) predawn Z + A level per Chl and aspects of D1 phosphorylation state. D1 phosphorylation state is given either as (a, b) the level of non-phosphorylated D1 (using SP1 antibody) or as (c, d) the level of D1 phosphorylation (using antiphosphothreonine antibody = anti-P-Thr). All data are from Fig. 3a and b for F_v/F_m; Fig. 3c and d for Z + A; Fig. 3e and f for phosphorylated and non-phosphorylated D1. Although correlations were observed for all (eight) sets of parameters in south- and north-facing needles, only those with r^2 above 0.7 are shown, i.e. a, b = southfacing needles; c, d = north-facing needles.

form at lower temperatures was suggested to favour thermal energy dissipation within the PSII core (Sane *et al*. 2002). The role of PSII core protein phosphorylation and zeaxanthin in the conversion of PSII cores to dissipating centres should be investigated further.

On cold winter nights, sustained phosphorylation of D1 (and, possibly, D2 and CP43) was not associated with phosphorylation of any LHCII proteins. This is consistent with the finding that there is a specific protein phosphatase for the PSII core proteins (Vener *et al*. 1999). One of the key proteins found to be involved in the regulation of PSII core protein phosphorylation is the inhibitor of PSII core protein phosphatase, TLP40 (Fulgosi *et al*. 1998). The strong increase of TLP40 in overwintering Douglas fir observed in the present study suggests that the change in PSII core protein phosphorylation plays an active role in winter acclimation.

In addition to the associated phenomena of sustained zeaxanthin-dependent energy dissipation and sustained D1 protein phosphorylation, an increase in the level of PsbS protein (Fig. 1) as well as in rapidly reversible energy dissipation was also apparent in response to winter conditions. This reversible dissipation can be assessed from the greater depressions in PSII efficiency at midday relative to predawn (Fig. 3A & B). Reversible dissipation is dependent on zeaxanthin and subject to regulation by the PsbS protein in concert with protons in the thylakoid lumen (Li *et al*. 2000, 2002). At present, it is unclear whether or not PsbS also plays a role in the sustained thermal dissipation (see, e.g. Savitch *et al*. 2002; Norén *et al*. 2003; Ensminger *et al*. 2004) that occurs in overwintering evergreens (for reviews, see Adams *et al*. 2002, 2004; Öquist & Huner 2003). We speculate that two populations of PSII core/LHCII units may coexist and shift in their proportions as the winter progresses, one with functional photochemistry and an increased capacity for pH/PsbS-dependent thermal dissipation and the other photochemically incompetent and with a high capacity for sustained thermal dissipation that is apparently pH-independent (Verhoeven *et al*. 1998).

Future research should address the role of specific proteins and the potential role of PSII core protein phosphorylation in the maintenance of thermal energy dissipation that does not reverse rapidly upon warming and occurs so frequently in many sclerophytic evergreen species during the winter. It is also important to address the environmental modulation of the regulatory system of PSII core protein phosphorylation and dephosphorylation, with a focus on TLP40.

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